From THE DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
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A THREE DIMENSIONAL SCAFFOLD FOR SINGLE STAGED TISSUE ENGINEERING

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A three dimensional scaffold for single staged tissue engineering

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By

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To my

Family and Friends

OVERVIEW

	Aims	M&M	Results	Conclusions
Study I	Evaluate a collagen-PCL scaffold with minced bladder mucosa *in vitro*	Minced tissue was cultured on top of the scaffold. Scaffold properties were evaluated.	Good proliferation. Multilayered epithelium after 4 weeks. High tensile strength.	Transplant with favorable properties for reconstruction of urogenital tract in one single-staged surgery.
Study II	Evaluate a collage-PLGA scaffold with minced bladder mucosa *in vitro*	Minced tissue was cultured on top and inside the scaffold. Scaffold properties were evaluated.	Good proliferation. Multilayered epithelium after 4 weeks. High tensile strength.	Transplant with favorable properties for reconstruction of urogenital tract in one single-staged surgery.
Study III	Evaluate differentiation of bone marrow MSCs into urothelium, separately or on top of a collagen-PCL scaffold	MSCs were co-cultured with urothelium or cultured with conditioned medium. MSCs were also differentiated on the scaffold.	MSCs differentiated into urothelial-like cells after 14 days with both methods, and on top of the collagen-PCL scaffold.	In the future, autologous bone marrow MSCs may be a source for urogenital regenerative medicine in cases with lack of native urothelial cells.
Study IV	Evaluate a collagen-PCL scaffold with minced skin in a rat model.	Minced skin was cultured on top of a collagen-PCL scaffold *in vitro* and *in vivo*, in a subcutaneous rat model. Scaffold properties were evaluated.	Good integration of scaffold. Keratinocyte proliferation on top of the scaffold that kept its tensile strength and elasticity.	Cell expansion on top of the scaffold could take place after transplantation *in vivo*. This may facilitate future urogenital reconstruction and autologous tissue expansion without *in vitro* cell culturing.

LIST OF SCIENTIFIC PAPERS

I. Fatemeh Ajalloueian, **Said Zeiai**, Ramiro Rojas, Magdalena Fossum, Jöns Hilborn.
One-stage tissue engineering of bladder wall patches for an easy-to-use approach at the surgical table.
Tissue Eng Part C, 2013, 19(9), 688-96.

II. Fatemeh Ajalloueian, **Said Zeiai**, Magdalena Fossum, Jöns G Hilborn.
Constructs of electrospun PLGA, compressed collagen and minced urothelium for minimally manipulated autologous bladder tissue expansion.
Biomaterials. 2014, 35(22), 5741-8.

III. Jixue Zhao*, **Said Zeiai***, Åsa Ekblad, Agneta Nordenskjöld, Jöns G Hilborn, Cecilia Götherström, Magdalena Fossum
*Shared first authorship
Transdifferentiation of autologous bone marrow cells on a collagen-poly(ε-caprolactone) scaffold for tissue engineering in complete lack of native urothelium.
J. R. Soc. Interface, 2014, 11(96).

IV. **Said Zeiai**, Clara I Chamorro, Jinxing Huo, Jöns G Hilborn, Magdalena Fossum.
The concept of In vivo tissue engineering as a single surgical procedure.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

BE	Bladder exstrophy
CK	Cytokeratin
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
H&E	Hematoxylin and eosin
HTX	Hematoxylin
MSC	Mesenchymal stem cell
PCL	Poly(ε-caprolactone)
PLGA	Poly(lactic-co-glycolide)
PVA	Poly(vinyl alcohol)
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscopy
UC	Urothelial cell
UP	Uroplakin
WB	Western blot

1 BACKGROUND

1.1 GENERAL

As a young surgeon, the most challenging situations I have encountered are when tissue is lacking. Treatment options when there is a lack of tissue due to different diseases and diagnosis are available in today's medicine. However, after for example severe trauma, surgical treatment of cancer, and congenital malformations there are sometimes limitations to our treatment options and the feasibility of treatment can be limited due to tissue shortage. Tissue engineering may lead to new solutions for these patients and give rise to better therapies and results.

Focus has been on the lower urinary tract particularly for children with congenital malformations. The urinary system consists of several organs in order to produce urine, transport the urine through the ureters to the bladder and store it in the bladder before elimination out of the body by the urethra. The function of the urinary organs is to maintain the body homoeostasis by regulating the water and ionic balance and at the same time achieve an active life with controlled continence (Rasouly and Lu 2013). We seek to find ways to engineer the bladder and urethra to restore the function of storing and eliminating urine in a controlled manner when there is a need of intervention.

1.2 EMBRYOLOGY OF THE URINARY TRACT

The cloaca is formed from the hindgut of the embryo, and is present up until week 7 of gestation. During week 7, the cloaca is divided by the urorectal septum into a ventral part, the urogenital sinus, and a dorsal part, which develops to form the anorectal canal. The urogenital sinus is usually divided into the cranial part or the vesical part, which will form the future bladder and the caudal part developing into the rest of the lower urogenital tract, such as the future urethra, prostate and external genitalia. The vesical part is connected to the allantois and functions in early fetal life as an outflow tract to drain the early developing bladder connected to the umbilical cord. The allantois regresses by week 15 of gestation and the bladder then separates from the umbilical cord. The remnant of the allantois, the urachus, is further elongated to form the median umbilical ligament. The posterior part of the vesicourethral canal develops into the pelvic parts of the urethra in the male and the full urethra in the female.

After 4-5 weeks of gestation the common nephric duct integrates into the lower part of the urogenital sinus where the future bladder neck will be situated. After the fusion, the orifice moves anteriorly and separates the common nephric ducts from the Wolffian duct. Further development of the bladder repositions the ureteric orifices, and with the internal urethral orifice they form a triangular area, which is their final position in the bladder wall called the trigone. Traditionally the trigone has been considered the only part of the bladder that is structurally different from the rest of the lower urinary tract, and recent studies have shown that it is most likely also derived from an endodermal layer.

In the early days of the bladder when it is still part of the urogenital sinus, it consists of bilayered cuboidal cells surrounded by undifferentiated mesenchymal cells. At about 12 weeks of gestation the mesenchymal bladder cells start differentiating into smooth muscle cells and by week 21 the bladder cells are similar to the full-grown bladder with 3-5 layers of urothelial cells and 3 layers of longitudinal and circular smooth muscle.

Bladder compliance is important for the function of the bladder, which is to store and empty urine at a desired time, although the formation and development of bladder compliance is mostly unknown.

The sphincters give rise to the continence function of the bladder and are developed in a similar manner in both females and males. The formation of the sphincters arises from a combination of the trigone, bladder detrusor and urethral sphincter complex. When the detrusor matures, it also forms a muscle band around the ureteric orifices and functions as the ureterovesical sphincters, which closes the orifices when the bladder contracts during voiding. This ostial-valve functions as an anti-reflux mechanism preventing the flow of urine back to the kidneys.

(Newman and Antonakopoulos 1989, Shafik 1996, Vize, Woolf et al. 2003, Yucel and Baskin 2004, Thomas, DeMarco et al. 2005, Viana, Batourina et al. 2007, Tanaka, Ishii et al. 2010, Rasouly and Lu 2013, Wein, Kavoussi et al. 2016)

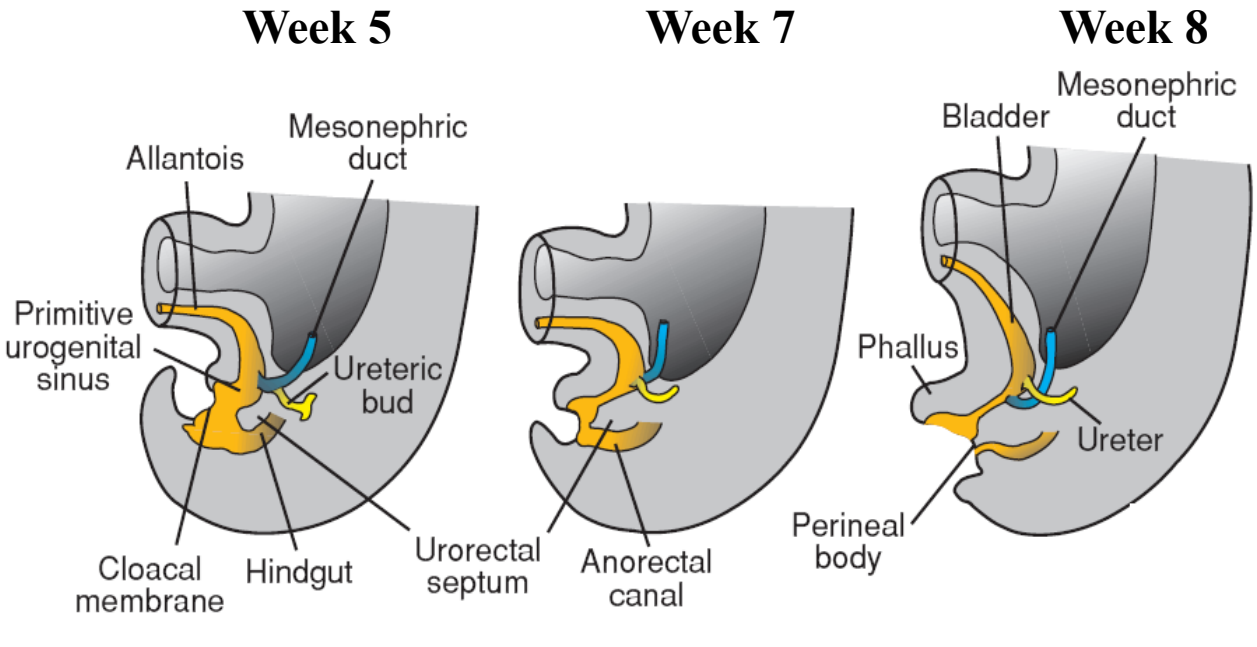

Figure 1: Development of the urogenital tract. Picture from www.slideshare.net, modified by the author.

1.4 BLADDER HISTOLOGY

The most organized part of the bladder is at the bladder neck consisting of three different layers, the inner longitudinal muscular layer, the middle circumferential or circular muscular layer and the external longitudinal muscular layer. The bladder neck is also used as the inner sphincter. The detrusor is arranged in the same manner but the layers are more distinct at the neck. In the trigone, there are two muscular layers, the superficial layer, which connects to the longitudinal urethral muscles and the deep muscle layer which connects to the detrusor.

Towards the luminal side of the bladder from the detrusor there is a connective tissue layer called the lamina propria, which connects the detrusor to the bladder mucosa consisting of urothelium. The lamina propria has vast microvasculature to support the epithelium. The bladder mucosa, the urothelium, is a transitional epithelium that consists of flat and cuboidal cells with the capacity to stretch and constrict so the bladder can function properly. The outer part of the cell lining consists of umbrella cells. These cells form an impermeable surface and an osmotic barrier to make sure the urine is not reabsorbed by the body. A layer of glycosaminoglycans covers the luminal surface of the umbrella cells, giving the cells a layer of protection from harmful substances and helping in the barrier function.

(Kingsnorth, Skandalakis et al. 2000, Woo, Hijaz et al. 2007, Fowler, Griffiths et al. 2008, Phull, Pan et al. 2011, Walters and Karram 2015)

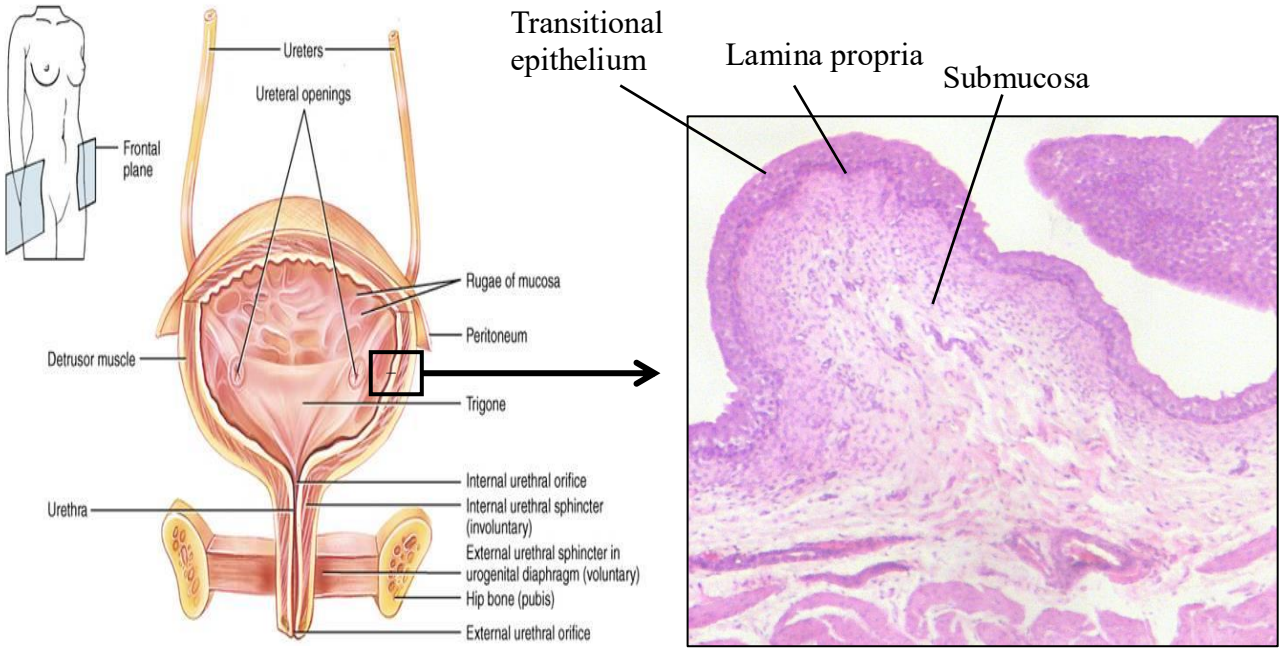

Figure 2: Anatomy of the female bladder (left), picture from www.slideshare.net and modified by the author. Histology of the urinary bladder (right), from Wikipedia, Urinary Bladder, CC BY-SA 3.0, modified by the author.

1.5 SKIN HISTOLOGY

The skin consists of two parts, the epidermis and dermis, and below there is a subcutaneous layer which consists of fatty tissue and larger blood vessels.

The epidermis consists of keratinized stratified squamous epithelium made up of four types of cells, keratinocytes, melanocytes, Langerhans cells and Merkel cells. Keratinocytes constitute over 90% of the epidermis and establish several layers as they ascend upwards to the surface. In the basal layer the keratinocytes are columnar cells separating dermis from epidermis. The next layer is the stratum spinosum where the keratinocytes develop desmosomes and attach to adjacent cells. In the third layer, stratum granulosum, the keratinocytes become squamous cells containing keratohyaline, which is a precursor to keratin. The last and most superficial layer, stratum corneum, is the most important layer and functions as a water repellant barrier. It consists of several layers of flattened cells without a nucleus, and contains the final keratin, which is a combination of keratohyaline and cytokeratin.

The dermis is separated from the epidermis by the basement membrane and consists of two layers, the papillary layer and the reticular layer. The most superficial layer of the dermis is the papillary layer and contains most of the nerves, sensory receptors and blood vessels. The reticular layer consists of dense collagenous connective tissue. The dermis provides the skin with its tensile strength and elasticity.

(Urmacher 1990, Kanitakis 2002)

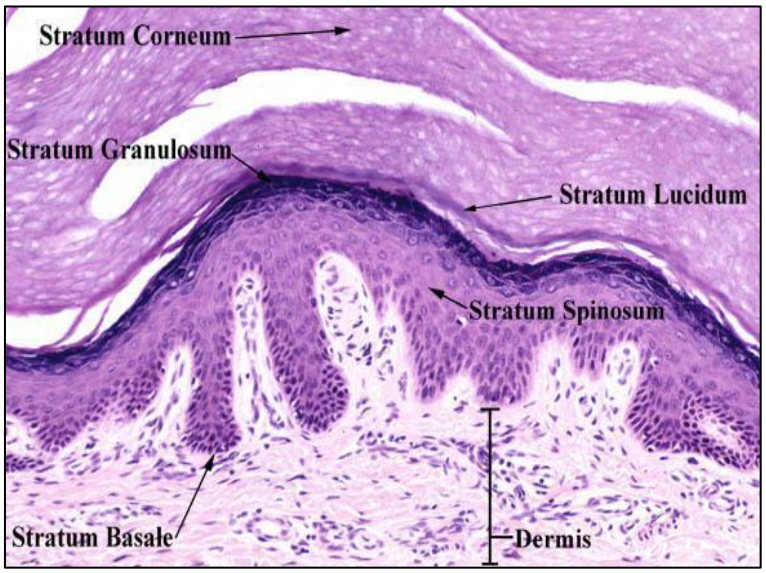

Figure 3: Basic skin histology. Picture from https://www.slideshare.net/moha_az/basic-skin-histology.

1.6 TISSUE ENGINEERING

The term tissue engineering which is also referred to as regenerative medicine, was first mentioned in 1985 when bioengineering pioneer Y.C. Fung of the University of California submitted a proposal to the National Science Foundation in the US for an Engineering Research Center Program award under the title "Center for the Engineering of Living Tissues". The first formal scientific meeting with the term tissue engineering was held in 1987 (Schultheiss, Bloom et al. 2000).

Tissue engineering is the merging of the clinical and scientific fields with engineering. Since the start of tissue engineering, the number of researchers and research papers within the field has grown exponentially. This has given rise to multidisciplinary cooperation across different fields and is today almost a requirement for successful research within the field of tissue engineering. Theoretical advantages when using autologous cells for tissue regeneration are: no foreign body reaction, grows with the patient, re-models according to local microenvironment and could potentially give rise to an unlimited number of cells.

The common way of tissue engineering with autologous cells is usually performed via several steps. In the first step, cells are harvested in different ways from the chosen organ or alternatively stem cells are harvested. The next step is to grow and cultivate the cells to the required amount and if there are stem cells they may need to be induced and differentiated into the intended cells. A scaffold resembling the organ of interest is produced and the cells are cultured within or on top of the scaffold. After the preparation of the scaffold is finished, a second surgical procedure is conducted and the transplant is put in place to expand or repair the intended organ (Chester, Balderson et al. 2004, Fossum, Svensson et al. 2007, Bowland, Ingham et al. 2015).

Today, tissue engineering of most organs is feasible and/or under clinical trials. For example, the skin, cartilage, vessels, bladder and urethra are some of the organs that have been investigated in human trials. The most common clinical tissue engineering application is currently performed in patients with burn injuries. Cultured sheets of human autologous keratinocytes have been used as a skin substitute in clinics for severe burn injuries, but this technique is still not widely used mostly because of the associated costs (Horch, Kopp et al. 2005).

There are problems facing the standard procedure of tissue engineering, including a long time for culturing (up to 7-8 weeks), the associated high cost, the requirement of advanced and highly specialized personnel and laboratories to conduct the process and the patient in addition must undergo two surgical procedures, for harvesting and later transplantation. To circumvent these problems, another idea behind tissue expansion is to guide the body to expand and regenerate tissue after a designed pattern using the body as an incubator. This would only require one surgical procedure and would bypass the downsides of traditional tissue engineering. This would, however, also require a higher standard during the initial operation and place especially high demands on the scaffold.

Meek introduced a theory in 1958 where he described how skin epithelium would regenerate from the edge of a cut surface of the skin by cell division and migration (Figure 4) (Meek 1958). This led to the idea that by cutting a piece of skin into two pieces, it would produce a longer boarder from where the cells can migrate and regenerate. Surgically minced tissue into sub millimeter parts has been shown to be able to repair a wound and give a 100-fold expansion of a skin graft (Hackl, Bergmann et al. 2012). In our group, we have also shown that it is possible to expand minced bladder mucosa to form a conduit to the bladder by a 3-fold expansion of the tissue (Reinfeldt Engberg, Lundberg et al. 2013). For these methods to be applicable on the bladder and/ or urethra a suitable scaffold would be needed with high tensile strength, that can be handled surgically, is biodegradable, and leads to a good proliferation of cells.

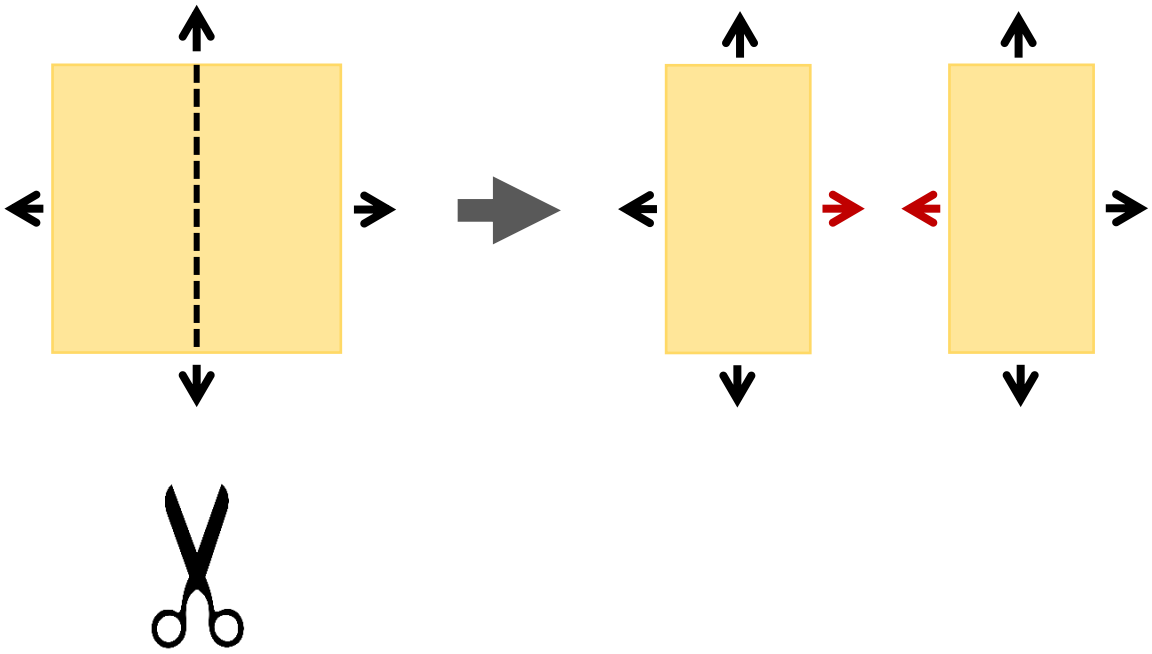

Figure 4: The theory of Meek. By cutting a piece of tissue into two pieces the total circumference increases and leads to a longer border from which cells can proliferate.

The scaffold may be the most important part of the steps in tissue engineering an organ and the success rate may depend on it. In bladder augmentation studies where a seeded PLGA copolymer scaffold was used, initial results showed a moderate benefit but a later phase-II trial had to be discontinued due to adverse effects, which included severe complications such as bladder perforation and bowel obstruction. Another smaller study using small intestinal submucosa showed better results, but without a clear clinical benefit. These results could be due to the scaffold properties or the lack of integration of the scaffold. In other organs, clinical implementations have also shown adverse effects, which could be related to the scaffold properties. Regeneration of skin with autologous sheets has shown poor results in

respect to mechanical stress. Clinical use of tissue engineered cartilage as well as arteries have also been associated with several scaffold-associated hurdles, such as lack of high tensile strength, lack of integration into the surrounding tissue, and lack of elasticity and compliance over time (Horch, Kopp et al. 2005, McBane, Sharifpoor et al. 2012, Joseph, Borer et al. 2014, Sloff, Simaioforidis et al. 2014, Bauge and Boumediene 2015, Bowland, Ingham et al. 2015).

When constructing a scaffold for the bladder there is a need for high tensile strength and elasticity, and the surgeon must also be able to handle the scaffold with ease. At the same time the scaffold should provide a beneficial environment for the cells to proliferate and migrate. Collagens are proteins that are part of the extracellular matrix in all species and provide good cell proliferation properties. The draw-back is that collagen has little mechanical strength and cannot be handled surgically. By low mechanical pressure it is possible to extract water from the collagen and thus reduce the volume more than 100 times. The phenomenon is called "plastic compression", and gives the collagen higher tensile strength. Despite the enhancement, the strength is unfortunately still too low for bladder augmentation purposes. (Brown, Wiseman et al. 2005, Martins, Filho et al. 2011) We have therefore investigated different polymers inside of our scaffold for additional strength and support. The first polymer was knitted Poly(ε-caprolactone) (PCL) which gives a higher compliance and degrades more slowly. The second polymer was electrospun poly(lactic-co-glycolide) (PLGA) which resembles the extracellular matrix and degrades at a faster rate. Both polymers are synthetic and biodegradable (Brown, Wiseman et al. 2005, Li, Mondrinos et al. 2006, Engelhardt, Stegberg et al. 2010, Woodruff and Hutmacher 2010).

Another issue that may arise when reconstructing an organ, or part of an organ, is the lack of cells. In traditional tissue engineering, the cells being used are from the targeted organ. In some cases, such as after severe trauma or after malignancy, cell harvesting may not be possible due to lack of cells or because of the risk of developing new malignancies. In these circumstances stem cells could be a possible solution. Mesenchymal stem cells harvested from the bone marrow are multipotent cells and can be induced into several lineages, such as osteoblasts, chondrocytes and adipocytes. They are easy to isolate and expand in culture. Mesenchymal stem cell transplantations have been in clinical use for over 15 years and several hundred patients have been treated without negative effects. For these reasons, we decided to also study these cells for bladder augmentation purposes (Petersen, Bowen et al. 1999, Pittenger, Mackay et al. 1999, Koc, Gerson et al. 2000, Sanchez-Ramos, Song et al. 2000, Le Blanc, Rasmusson et al. 2004, Oswald, Boxberger et al. 2004, Bernardo, Zaffaroni et al. 2007, Prockop, Brenner et al. 2010, von Bahr, Batsis et al. 2012).

1.7 BLADDER EXSTROPHY

Bladder exstrophy is part of the extrophy-epispadias complex and is a severe form of an abdominal midline malformation. Structures involved in the malformation include the abdominal wall, the urinary system, the genitalia, the pelvis, the pelvic floor and could also involve the spine and the anus, as in cloacal malformation, the most severe form.

The prevalence of the malformation varies in the published data and ranges from 2-4 per 100 000 live births. The male to female ratio has been reported as 1.14-6:1 and caucasian infants seem to have a higher prevalence overall.

The new-born child with bladder exstrophy presents with an open bladder of varying size in the lower midline of the abdomen. The ureteric orifices are visible and dripping urine. The bladder mucosa is affected and mucosal polyps may be present. If closure has been delayed further signs of inflammation may be present. Rectus diastasis is always present and bilateral inguinal hernias are present in most patients.

The genitalia of the patients are affected differently depending on the gender. In the new-born male, the urethral plate is open (epispadic) and covers all the dorsal portion of the penis, which puts both corpora cavernosa below the urethral plate. The penis is dorsally curved and is shorter than normal. In the female child, the clitoris is split around an open urethral plate, the perineum is shortened and the vagina is narrower than normal. There is also a wide pubic diastasis and a rotational displacement of the pelvis in both gender.

There are different ways to manage the malformation. The golden standard is functional reconstruction. Earlier, a much simpler method was to remove the bladder and perform a urinary diversion and, later, a sigmoid pouch. Functional bladder reconstruction can today be performed in one, two or three staged concepts. These concepts involve, at different time points in age, the closure of the bladder, urethral closure, the closure of the abdominal wall and later epispadias repair. The surgeon may also perform pelvic osteotomies during the primary closure to deepen the flattened pelvis and by doing so also release the tension in the abdominal wall. There is no consensus on the best time of surgery and whether to perform an osteotomy.

Retrospective data has shown a continence rate of 80% in childhood, but after childhood the number of patients who need a bladder augmentation, a bladder neck plasty or self catherization increases. The continence rate also decreases after a redo bladder neck plasty to around 40%. If the bladder neck resistance is too low, different injectable materials can be used to increase the urethral resistance. Other complications associated with bladder exstrophy are vesicoureteric reflux, bladder spasm, stone formation and urinary tract infections. If bladder reconstruction fails and the reservoir is too small, bladder augmentation can be performed with parts of the bowel, preferably the sigmoid or ileum. Bladder augmentation however, has its own complications such as infections, increased mucus production, metabolic disturbances, perforation, stone formation and, in addition, a higher risk of malignancy. There are today no ideal treatment options with a low risk of complications.

(Sponseller, Bisson et al. 1995, Grady, Carr et al. 1999, Baka-Jakubiak 2000, Greenwell, Venn et al. 2001, Nelson, Dunn et al. 2005, Thomas, Duffy et al. 2008, Ebert, Reutter et al. 2009, Gearhart, Rink et al. 2010, Reinfeldt Engberg, Mantel et al. 2016)

Figure 5: 4 week old girl with bladder exstrophy.

1.8 HYPOSPADIAS

Hypospadias is a penile congenital malformation where the urethral opening is located ectopically on the ventral side of the penis. The urethral opening can be positioned proximal to the scrotum or perineum. Hypospadias is also associated with chordee, a ventral curvature of the penis, and a dorsally placed foreskin. The chordee is usually found in the more proximal hypospadias. In many cases the scrotum is located cranially leading to a penoscrotal transposition.

Hypospadias is a common malformation and has a prevalence of around 1 in 125 boys in Sweden and the United States. Several studies show that the prevalence has increased and is not due to only better surveillance systems and a shift in diagnostic criteria (Manson and Carr 2003, Nordenvall, Frisen et al. 2014).

Many classifications of hypospadias have been proposed, but one of the most widely used was described by Barcat and modified by Ducket. This classification is based on the placement of the meatus and called anterior hypospadias (distal of the sub-corona), middle hypospadias (on the penile shaft) and posterior hypospadias (penoscrotal to perineal position) (Horton 1973, Walsh and Campbell 2002).

Figure 6: Different types of hypospadias. The meatus is located at an abnormal position. CC-SA-1.0.

The objectives of the surgical correction of hypospadias are to give the boy a straight penis with acceptable sexual function, to be able to void with a normal flow, and satisfactory cosmetic results where the meatus is slit-shaped and placed distally on the glans.

The history of hypospadias surgery goes back all the way to ancient Greece where partial penectomy was performed. Since then, advancements have focused on enhancements in function and cosmetics. In the 19th century the most significant advances occurred such as urethroplasty, preputial skin flaps, and multilayered closures. Technical advances in the 1980s and 1990s led to better appearance and function of, particularly, distal hypospadias.

The success rate for distal hypospadias is over 90% when excluding adult patients and redo surgeries. For proximal hypospadias, the situation is different, and several recent studies have shown a complication rate as high as 50-70%, which is higher than the historically lower complications rate of 15-30%. This may be due both to longer follow-up times and a larger number of patients. Even though many of these patients only require one redo surgery, some of them must undergo several and complicated surgeries which leads to high morbidity. Although most patients are straight forwardly treated, some are complicated with a severe lack of tissue, especially in redo surgery. Different tissue substitutes have been used, such as buccal mucosa and preputial skin flaps, but these are also associated with high complication rates.

(Duckett 1981, Snodgrass 1994, Barbagli, Sansalone et al. 2012, Fine, Reda et al. 2015, Stanasel, Le et al. 2015, Long and Canning 2016)

1.9 AIM

The aim of this thesis was to develop a scaffold suitable for tissue engineering with the proper characteristics when treating patients with lack of tissue in the lower urinary tract. The scaffold should have a high tensile strength, in the same range as the urinary bladder, be compliant, biodegradable, and easy to construct and provide cells with a satisfactory

environment to proliferate and create new tissue. We sought to study our transplants both *in vitro* and *in vivo*. We also wanted to explore the possibilities of using stem cells as a cell source when engineering a bladder transplant, so as to also be able to provide a solution for patients lacking autologous urothelial cells.

2 MATERIALS AND METHODS

2.1 PCL (STUDIES I, III, IV)

We used PCL (Sigma-Aldrich) with an average molecular weight of 80,000 g/mol which was dried in a vacuum and later compressed into cylindrical rods. Under a nitrogen atmosphere the rods underwent melt spinning at 180 °C. The filaments where drawn at 30 °C and subsequently knitted using a Lawson Hemphill FAK-sampler, which has a cylinder of an 89 mm diameter with 380 needles. Air at a pressure of 40 psi (3 bar) and dry and clean instruments were used during the knitting process. By knitting the PCL filaments, a higher elasticity is achieved.

The PCL surface had to become more hydrophilic for better attachment to the collagen. For this purpose, the PCL surface was preactivated with an alkaline hydrolysis. The PCL mesh was put in 2.5 M NaOH for 40 min at 40 °C, rinsed with distilled water, and placed in PVA (Merck; 72,000 g/mol) 1% w/v solution for 10 min. Before use, the PCL-knitted mesh was sterilized in 70% ethanol for 30 min.

2.2 PLGA (STUDY II)

Preparation of the PLGA solution (20% w/v) (poly(D,L-lactide-co-glycolide); a lactide:glycolide ratio of 75:25; molecular weight 66,000e107,000 g/mol) was carried out by dissolution in a dry chloroform (Sigma)/DMF(Sigma) mixture under magnetic stirring for 3 h at 50 °C. Electrospinning was carried out by a feed rate of 1 mL/h and a voltage of 16 kV applied to a 21 gauge blunt needle tip of a 1 mL syringe filled with the PLGA solution. By applying it onto a PCL winded tube on the collecting mandrel we acquired the “optimized” PLGA mat and, by applying it directly on to the collecting mandrel, we acquired the “ordinary” PLGA mat. The rotational speed was 200 rpm, the distance between the collector and the needle was 15 cm, the humidity was 50% and the maintained temperature was 24 °C. The electrospun mats were vacuum dried for 24 h after being separated from the collector and the winded tube. By spinning the PLGA we tried to mimic the extracellular matrix.

2.3 CONTACT ANGLE MEASUREMENTS (STUDY I)

A Video-Based Optical Contact Angle Meter (Data-Physics OCA 15EC) was used to investigate the hydrophilicity of the alkaline-hydrolyzed and PVA-treated PCL meshes. Pieces 4 cm^2 were attached on a microscope slide with tape. 6 µL of drop was measured automatically while the stage with the slide moved up to put the droplet on top of the sample surface. Before the measurement, the drop was left to reach equilibrium and using the SCA 10 software the contact angles of both sides of the drop were calculated.

2.4 PREPARATION OF THE SCAFFOLD (STUDIES I-IV)

Rat-tail collagen type I solution was used (2.06 mg/mL protein in 0.6% acetic acid; First Link Ltd.) by mixing 4.8 mL of collagen type I with 0.6 mL of 10X Eagle’s Minimum Essential

Medium (Invitrogen, Sweden), afterwards neutralized with 2.5 M NaOH and finally 0.6 mL of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Sweden) was added to the mixture. A rectangular or ring-shaped mold was used where one third of the solution was poured into the mold and incubated at 37 °C for 10 min to form a semi-rigid gel. The sterilized PCL mesh or PLGA mat were put on top of the collagen and the rest of the collagen solution was cast upon the polymers. After this, the scaffold was once again put into the incubator for the completion of the gel formation.

2.5 PLASTIC COMPRESSION (STUDIES I-IV)

Plastic compression was performed on the gel-polymer constructs. The gels were transferred to blotting elements consisting of, on the bottom, three sterile gauze pads, on top of these a 400 µm thick stainless steel mesh with mesh sizes of 200 µm, and above that a sterile nylon mesh with a mesh size of 40 µm. The gel-polymer construct was covered with another piece of nylon mesh, steel mesh, and on top a loading plate. The load was approximately 120 g and was left at room temperature for 5 minutes. With the help of the loading plate water was pushed down into the gauze pads and reduced the thickness of the construct from 1 cm to under 0.5 mm, thus finalizing the scaffold.

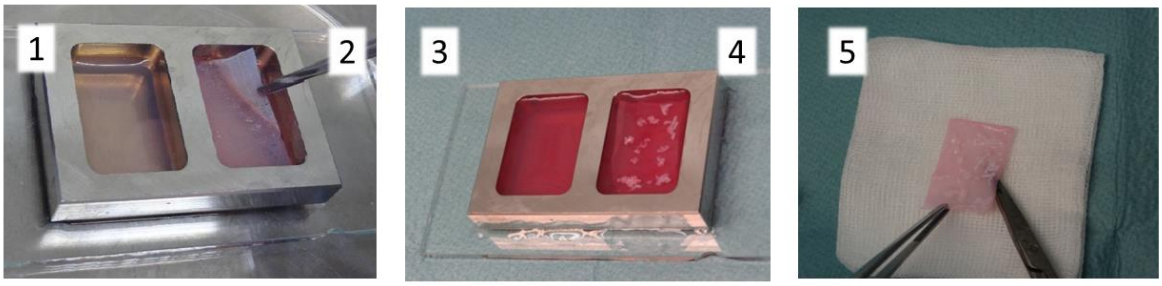

Figure 7: Preparation of the scaffold. A slab of collagen is poured into the mold and incubated for 10 min (1). The PCL mesh or PLGA mat is put on top (2) and collagen is poured on top (3) and incubated for another 20 min. Minced tissue is placed on top of the scaffold (4) and plastic compression is performed finishing the transplant (5). Adapted from Scientific paper I with permission.

2.6 MINCED TISSUE (STUDIES I, II, IV)

When preparing the minced tissue from the bladder, the bladder was washed and rinsed in DMEM and the mucosa was removed mechanically with scissors. A piece 10x10 mm was cut out using a mincing device to produce particles measuring approximately 0.3x0.8 mm. When skin was used, everything apart from the epidermis and upper part of dermis was removed and the mincing was performed in the same manner.

For seeding the hybrid scaffold, the minced particles were placed on the gel-polymer construct before plastic compression. We opted for a 1 to 6 expansion rate as a proof of concept and used the equivalence of 1 cm^2 of minced tissue on top of 6 cm^2 of scaffold. We believe, however, that a higher expansion rate would be possible.

2.7 CULTURING AND CHARACTERIZATION OF MESENCHYMAL STEM CELLS (STUDY III)

Bone marrow was harvested and diluted with phosphate-buffered saline (PBS) to a concentration of $1x10^7$cells/mL. By density gradient centrifugation on Percoll (Amersham, Biosciences, Uppsala, Sweden) mononuclear cells were collected and suspended in DMEM-low glucose (Invitrogen) with 10% fetal bovine serum (FBS, Sigma) and 1/100 antibiotic antimycotic solution (Invitrogen). The collected cells were plated at a density of $1.6x10^5$ cell/cm^2 and cultured at 37 ^{o}C in humidified air containing 5% carbon dioxide. The medium was changed every 3-4 days and passaged at 70% confluence to a density of $4x10^3$ in culture flasks. For our experiments we used cells at passage 3-5. Differentiation to adipogenic, chondrogenic and osteogenic cells was established and we also tested for surface expression of CD14, CD29, CD31, CD34, CD45, CD73, CD80, CD90, CD105, CD166 and HLA class I and class II as previously described (Le Blanc, Tammik et al. 2003).

2.8 HARVESTING AND CULTURING UROTHELIUM (STUDY III)

Urothelial cells were harvested by bladder washing with a method developed by our group (Fossum, Gustafson et al. 2003). In short, the bladder is accessed by a catheter through the urethra and is washed with sterile saline a couple of times using a syringe. The procedure is repeated until approximately a total amount of 300 mL has been collected. The saline containing the cells is then centrifuged at 500 g and the cell pellet resuspended in DMEM (Invitrogen).

The urothelial cells, as well as in vitro cultured transplants containing minced tissue were cultured in epithelial cell medium, containing DMEM and Ham's F12 (Gibco) in a four to one mixture. Other ingredients included were: 10 % fetal bovine serum (Gibco), insulin (5 µg/mL), hydrocortisone (0.4 µg/mL), adenine (21 µg/mL; Sigma), cholera toxin (10^{-10} mol/L), triiodothyronine ($2 \cdot 10^{-9}$ mol/L), transferrin (5 µg/mL), epidermal growth factor (10 ng/mL), and antibiotics (50U/mL penicillin and 50 µg/mL streptomycin). Cells were maintained at regular incubator settings of 5% carbon dioxide and humidified air at 37 ^{o}C.

When culturing urothelial cells from bladder washings, epidermal growth factor was added after 24 h of culture. The cells media were changed three times a week. After two to three weeks the cell colonies were passaged to a 25 cm^2 flask (passage 1), but we only used urothelial cells from passage 2 and onwards in our experiments.

2.9 INDUCTION OF MESENCHYMAL STEM CELLS TO UROTHELIAL CELLS (STUDY III)

Mesenchymal stem cells from the third passage were used. By using conditioned medium in two different ways, mesenchymal stem cells were induced into urothelial cells. The exposure to conditioned medium was 14 days with both methods.

In the first method, mesenchymal stem cells were cultured with conditioned medium gathered from urothelial cells. When the urothelial cells where at 70-80% of confluence their medium

was collected and removed from any cells through centrifugation and filtered through a mesh of 0.2 µm pore size. Consequently, DMEM (Invitrogen) with 10% FBS (Sigma) was added to the gathered medium in a four to one ratio. As controls, mesenchymal stem cells where used cultured with fresh filtered medium.

In the second method, mesenchymal stem cells (~3000 cell/cm^2) were co-cultured indirectly with urothelial cells (~7000 cell/cm^2) separated by a barrier membrane of 0.4 µm in pore size (24 mm dish, Transwell, Corning 3412). As controls, mesenchymal stem cells cultured in the same manner but with allogenic mesenchymal stem cells were used.

2.10 UROTHELIAL GENE AND PROTEIN EXPRESSION (STUDY III)

By using RT-PCR, specific gene transcripts can be measured. A specific RNA is changed to its DNA complement by reverse transcriptase and, subsequently, the new cDNA is exponentially amplified several orders of magnitude before detection. After 14 days of induction the differentiation of mesenchymal stem cells into urothelial cells was determined by measuring specific gene transcripts by RT-PCR. Using the RNAeasy mini kit (Qiagen) RNA was isolated from the cells. We synthesized cDNA by using the cDNA synthesis kit (Thermo Scientific) and analyzed transcripts by using the three different PCR conditions; 94°C for 30 s, 55 °C for 30 s and 72 °C for 30 s during 35 cycles. A 10 min extension step was conducted for all our PCR experiments. Using a 1.5% agarose gel the reaction products were analyzed by electrophoresis. For internal control, human glyceraldehyde-3-phosphate dehydrogenase was used.

Western blot is a method to detect a specific protein by attaching antibodies and identify the protein by gel electrophoresis. Protein expression for differentiation to urothelial cells was assessed after 14 days by Western blot. Mesenchymal stem cell lysates were used for western blot for urothelial lineage-specific gene expression. Cells were washed with PBS, and homogenized in tissue protein extraction reagent with halt protease inhibitor cocktail (Thermo Scientific) and thus lysates were obtained. Using SDS-PAGE (15%, Bio-Rad) the same amount of protein was separated and moved to a polyvinylidene difluoride membrane (GE Healthcare). Unspecific binding was blocked with 5% non-fat dry milk and the membrane was incubated with primary antibodies overnight at 4 °C. Thereafter, blots were stained with anti-mouse IgG conjugated to horseradish peroxidase (1:2000, Dako) and assessed with an enhanced chemiluminescence system (GE Healthcare).

2.11 HISTOLOGY AND IMMUNOHISTOCHEMISTRY (STUDIES I-IV)

For morphological studies, either routine staining or specific protein staining is used to characterize the cells. Antibodies attach to a specific protein, which can be stained and observed.

All samples containing our scaffold were fixed in formaldehyde solution and dehydrated before being embedded in paraffin and cut into 4 µm thick sections. The slides were rehydrated and stained with Hematoxylin and Eosin for routine histology.

Protein markers were also investigated in our studies. We used the Vectastain elite ABC kit (Vector) for immunohistochemistry of our slides. Inactivation of endogenous peroxidase was performed with 0.3% hydrogen peroxidase for 15 min. The slides were washed with PBS, the primary antibody was added, and the slides were incubated at 4 °C in a humidified chamber overnight. After washing with PBS, the samples were incubated with diluted secondary antibody for 30 min at 37 °C, washed again, and incubated with Vectastain elite ABC reagent for 30 min. Lastly, peroxidase substrate solution was added to the slides and incubated until a sufficient stain intensity developed, before being counterstained with hematoxylin.

We used several antibodies to assess the expression of proteins in the cells for the evaluation of cell type and proliferation activity. MNF-116 and C11 react with many cytokeratins that are found in the intracytoplasmic cytoskeleton of epithelial tissue. MNF-116 reacts to cytokeratin 5, 6, 8, 17 and 19, and CK11 reacts to cytokeratin 4, 5, 6, 8, 10, 13 and 18. UpIIIa is more specific to urothelial cells and is expressed in the superficial umbrella cells of urothelial cells. CK-18 is present in the urogenital tract, but also in other locations such as the respiratory and digestive epithelium.

2.12 TENSILE STRENGTH (STUDIES I,II AND IV)

We used the Instron 5944 tensile tester with a load of a 50-N load-cell with a crosshead speed of 10 mm/min and a gauge length of 40 mm under wet conditions in studies I and II. All samples were prepared by incubating in PBS for 24 h at 37 °C. Five measurements were performed at each time point.

In study IV a Shimadzu Autograph ASG-X tensile machine with a 10-N load cell was used. Specific fixture was used to clamp the sample and to remove the sample slipping. The gauge length of the implant samples was 10-12 mm with a width of 10 mm. With these settings, the maximal measurable tensile strength was approximately 2.4 MPa, which is in the same magnitude as the normal bladder. (Martins, Filho et al. 2011) Five measurements were performed at each time point.

2.13 ELECTRON MICROSCOPY (STUDY I AND II)

All the samples were also evaluated by scanning electron microscopy (SEM). After fixation in formalin for 1 h, the specimens were washed with distilled water and then passed through a graded ethanol series to remove the water. Afterwards, the specimens were put for 15 min in hexamethyldisilazane (Sigma-Aldrich), air dried, mounted on stubs and coated with gold before examination in a SEM (Zeiss 1550).

2.14 IN VIVO EXPERIMENT (STUDY IV)

Male rats (Sprague Dawley) were put in general anesthesia in a box with sevoflurane until asleep and then placed on the operating table and kept anesthetized with sevoflurane using a mask during the whole procedure.

The back of the rat skin was waxed with Veet Easy Wax Removal Strips (Reckitt Benckiser, Slough England) and sterilized with Sterillium (Hartmann, Heidenheim, Germany). A 1 cm^2 full skin biopsy was taken from the rat and the lower dermis and the subcutaneous parts were removed with scissors leaving only the upper dermis and epidermis (approximately 0.8 mm thick). A mincing device was used to cut the upper dermis and epidermis into smaller pieces horizontally and vertically to approximately 0.3x0.3 mm pieces. The minced tissue was prepared at the same time as the scaffold. Minced skin tissue was distributed on top of the scaffold in a 1:6 expansion rate and controls were made without any tissue. Plastic compression was performed and scaffolds with or without minced skin were sutured subcutaneously on the left or right side of the back of the rat with three single stitches using Ethilon 4-0 (Ethicon, Somerville, NJ). For each experiment, one control without minced tissue was sutured on the contralateral side of the back of the rat. The distribution between right and left side was randomly chosen between tissue-loaded scaffold and control. The wounds were closed with Ethilon 4-0 (Ethicon, Somerville, NJ) running sutures. A bandage was placed on the wound and the rats were observed until awake. All rats were kept in cages with two to three rats in each. After five days, ten days, and four weeks the rats were euthanized and samples were taken out for examination.

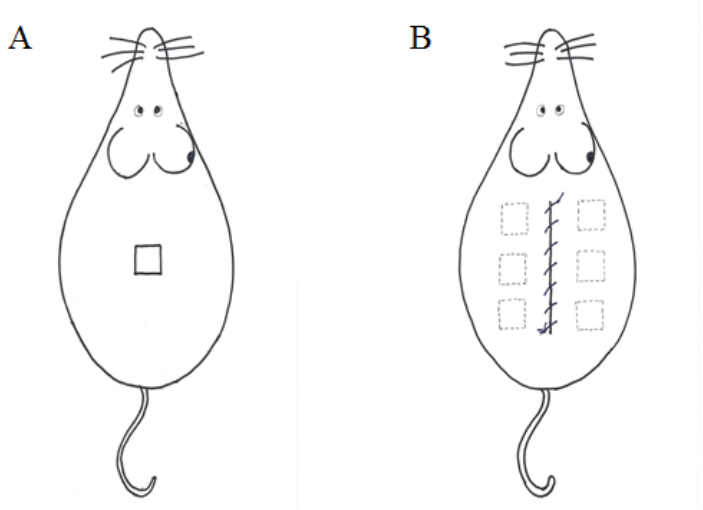

Figure 8: A: Schematic drawing demonstrating the position of the 1x1 cm biopsy on the back of the rat skin after depilation. B: Same rat after subcutaneous implantation of scaffolds with or without minced skin (squares with interrupted lines) and central wound sutured with continuous sutures. Cartoon drawn by Magdalena Fossum.

2.15 ETHICS

For ethical reasons, we try as far as possible to conduct studies *in vitro* to lower the amount of *in vivo* trials. However, before the developed method can be conducted in a human trial animal studies must be performed. By optimizing the *in vitro* trials, fewer animals will be needed, and by also refining the method when performing animal studies, we will have a more reliable treatment going into human trials. If possible, we always use smaller animals before large animal models if the same information can be gathered. We also try to keep the morbidity and mortality as low as possible in accordance with veterinary supervision. Before the start of our animal experiment ethics permission was approved by the Swedish Board of Agriculture.

The regional ethics committee in Stockholm approved harvesting of bone marrow in adults, and informed consent was obtained from all donors. The ethics committee of the hospital approved cell harvesting of urothelial cells from children during surgery after informed consent was given by the parents.

3 RESULTS

3.1 STUDY I

3.1.1 PCL mesh

After only alkaline hydrolysis the water contract angle on the PCL mesh declined from 104 ±5.3º to 79 ±6.9º, but was not stable over time. By performing hydrolysis followed by a PVA adsorption we achieved a stable result and an even greater hydrophilicity with a contact angle of 41 ±11.2º (Figure 8).

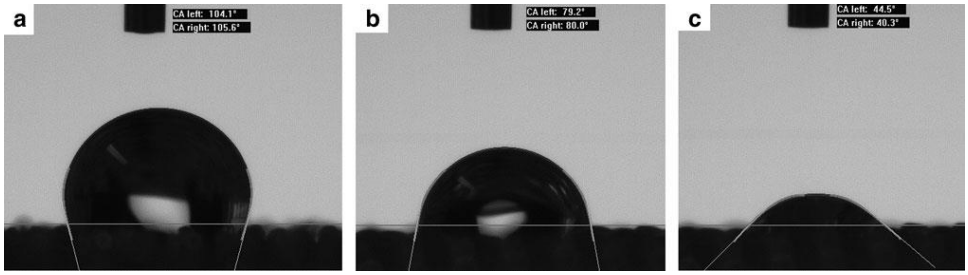

Figure 9: Water contact angle measurement, showing how hydrophilic properties increase. The angle decreased from approximately 104º (a) to 79º after alkaline hydrolysis (b) and was further lowered to 41º after subsequent PVA treatment. Adapted from Scientific paper I with permission from the publisher.

After using the hydrophilic PCL-knitted mesh, we achieved a stable integration with the collagen and disintegration did not occur during handling of the construct or during the culturing period. In the microscale, we observed a nanofibrous structure with an average diameter of 47 ±6 nm, which is comparative to that of native extracellular matrix (Mo, Xu et al. 2004). When removing the collagen from the PCL surface we could see collagen threads in between the PCL mesh (Figure 9). In contrast when non-treated PCL was used, spontaneous delamination of the collagen occurred and no anchoring of collagen could be seen within the PCL mesh.

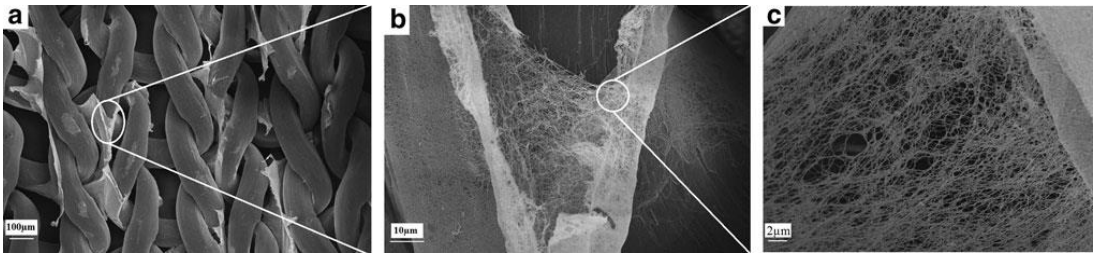

Figure 10: SEM images showing the integration of collagen within the PCL-knitted mesh, after the collagen has been removed (a). Higher magnification of residual collagen in between PCL fibres (b). Further magnification of collagen fibres resembling extracellular matrix (c). Adapted from Scientific paper I with permission from the publisher.

3.1.2 Tensile strength

Tensile strength of our hybrid construct was 17.9 ±2.6 MPa compared with 0.6 ±0.12 MPa for the collagen only. Mechanical handling of collagen or plastic compressed collagen was not possible due to low stability. When culturing on only plastic compressed collagen without a PCL mesh, we observed a dimensional change or rupture of the transplant.

3.1.3 Cell-seeded PCL-collagen scaffolds

After four weeks of culturing with minced mucosa, phase-contrast microscopy showed that the cells had migrated from the minced tissues and spread on top of our construct. SEM images visualized a mostly confluent epithelial sheet after two weeks and fully confluent layer after four weeks and multi-layered epithelium after six weeks. Histological staining confirmed the results at two, four and six weeks. H&E showed a single layer epithelium after two weeks and multi-layered epithelium already after four weeks. Cells were also present inside the scaffold after six weeks indicating a remodelling of the collagen. Immunohistochemistry showed cells positive for Ki-67, indicating proliferation in all samples. Cells were also positive for MNF-116 indicating epithelial origin.

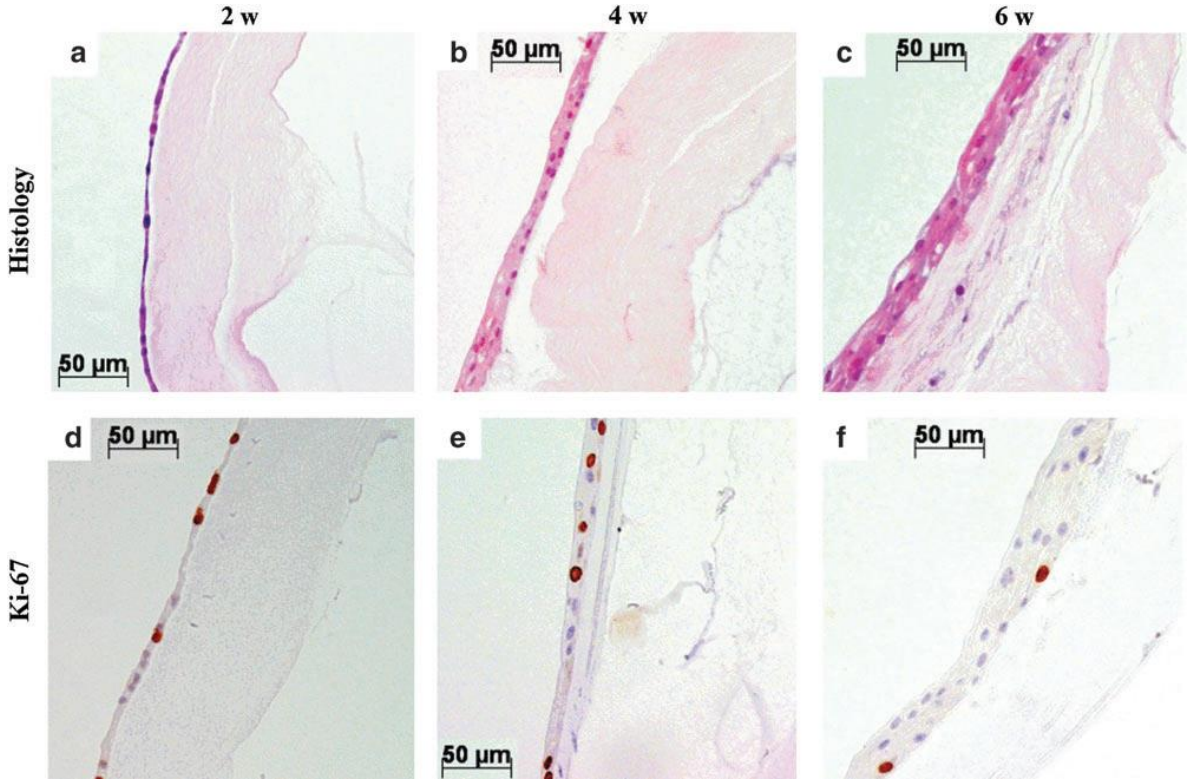

Figure 11: Routine histology of the epithelium on top of the collagen (a-c). After six weeks, we found a multi-layered epithelium and also cells inside the collagen (c). Brown nuclei cells stained for Ki-67, indicating proliferative cells throughout the experiment (d-f). Adapted from Scientific paper I with permission from the publisher.

3.2 STUDY II

3.2.1 Characterization of the PLGA scaffold

We compared the optimized PLGA mat with the ordinary through SEM images. The optimized mat contained altering sections of denser ordinary parts and parts with broadened fiber spacing. The average pore diameter in the ordinary PLGA was calculated to 7.54 µm compared with 24.12 µm in the optimized samples. Plastic compression resulted in collagen fibers with an average diameter of 43 nm, compared with the PLGA fiber diameter of 705 nm on average. The hybrid scaffold resulted in a good integration between the collagen and PLGA fibers, and collagen fibers could be seen between the PLGA fibers after plastic compression.

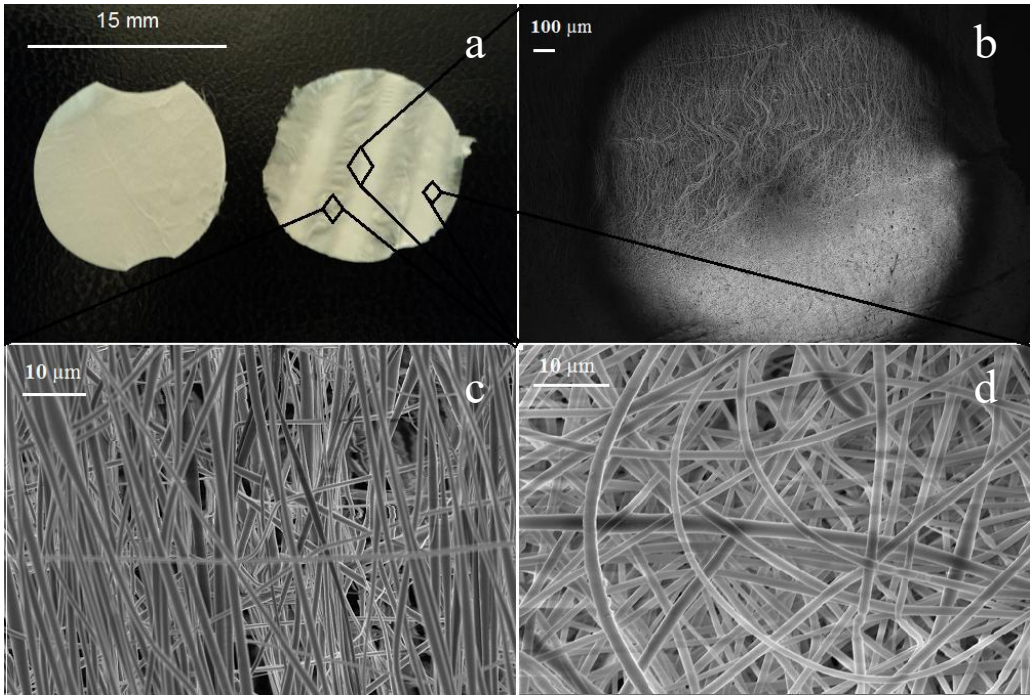

Figure 12: Ordinary PLGA scaffold (a, left) and optimized PLGA scaffold (a, right). The optimized scaffolds demonstrated a clear difference between two different textures (b), as shown in higher magnification of the porous parts (c) and the ordinary parts (d). Adapted from Scientific paper II with permission from the publisher.

3.2.2 Tensile strength

The tensile strength of the collagen was, as previously noted, 0.6 ±0.12 MPa and increased six-fold to an average of 3.57 ±1.1 MPa for the hybrid collagen-PLGA scaffold. The stiffness and elasticity also increased from 22.7 MPa and 5% elongation at break for plastic-compressed collagen to 45.5 MPa and 81% elongation at break for the PLGA-collagen construct.

3.2.3 Cell-seeded PLGA-collagen scaffolds

The placement of the minced tissue either on top or on top and inside the collagen did not affect the outcome. With both methods, we observed a single layer epithelium covering the scaffold after two weeks and a multilayer epithelium after four weeks. All cells covering the scaffold had the morphological characterization of urothelium. Immunostaining of the cells for cytokeratins with MNF-116 confirmed the epithelial origin and showed a typical transitional urothelium (figure 13). Staining for Ki-67 indicated cell proliferation in all samples during the whole experiment (figure 13).

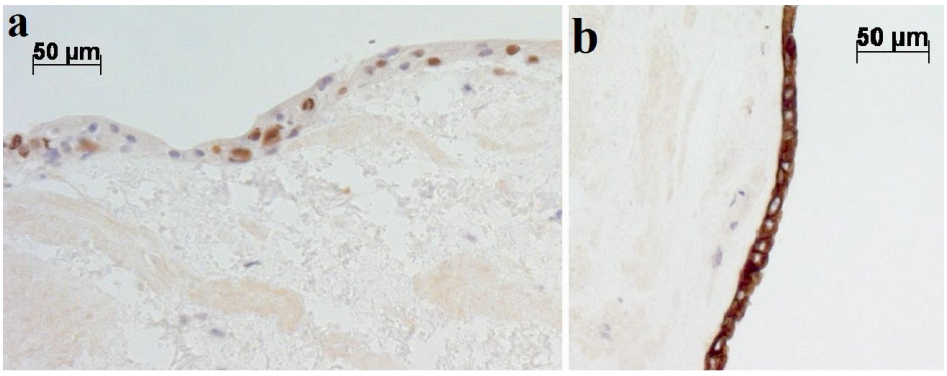

Figure 13: After four weeks in culture cells were positive for Ki-67, indicating a proliferative state (a). Cells were also positive for MNF-116, indicating an epithelial origin (b). Adapted from Scientific paper II with permission from the publisher.

3.3 STUDY III

3.3.1 Characterization of mesenchymal stem cells

MSCs were successfully isolated, expanded and cultured. They presented a phenotype with typical mesenchymal spindle-shaped morphology. When the MSCs were induced in adipogenic conditions, the cells changed morphology and produced intracellular droplets positive for oil red O staining. When instead being cultured for three weeks in osteogenic medium, they could deposit abundant mineralized matrix positive for alizarin red S staining and Von Kossa black staining. The cells demonstrated a typical antigen pattern with flow cytometry, fulfilling the criteria for MSCs (Le Blanc, Tammik et al. 2003). For more than 90% of the cells, antigen expression was positive for CD73, CD90, CD105 and HLA class I and positive for less than 5%, i.e. negative, for CD14, CD31, CD34, CD45, CD80 and HLA class II.

3.3.2 Characterization of urothelial cells

Urothelial cells obtained from bladder washings formed colonies and expanded in a monolayer, and had typical urothelium growth characteristics and morphology. We confirmed the urothelial phenotype with immunostaining for cytokeratin and uroplakin.

3.3.3 Induced mesenchymal stem cells

The mesenchymal morphology changed after 14 days of induction, from spindle-shaped and non-colony-forming into a polygonal epithelium-like shape resembling urothelial cells. The results were similar with both methods, either cultured with conditioned medium or when mesenchymal stem cells were co-cultured with urothelial cells. None of the mesenchymal stem cells had the morphology associated with the previous characterization of adipogenic or osteogenic lineages. In our control groups the morphology did not change.

Immunostaining of induced mesenchymal stem cells was positive for specific urothelial markers such as CK-18 and UpIIIa, but was not positive in the controls. We estimated 90% of the cells were positive for the markers and were believed to have differentiated into a urothelial lineage (Figure 13).

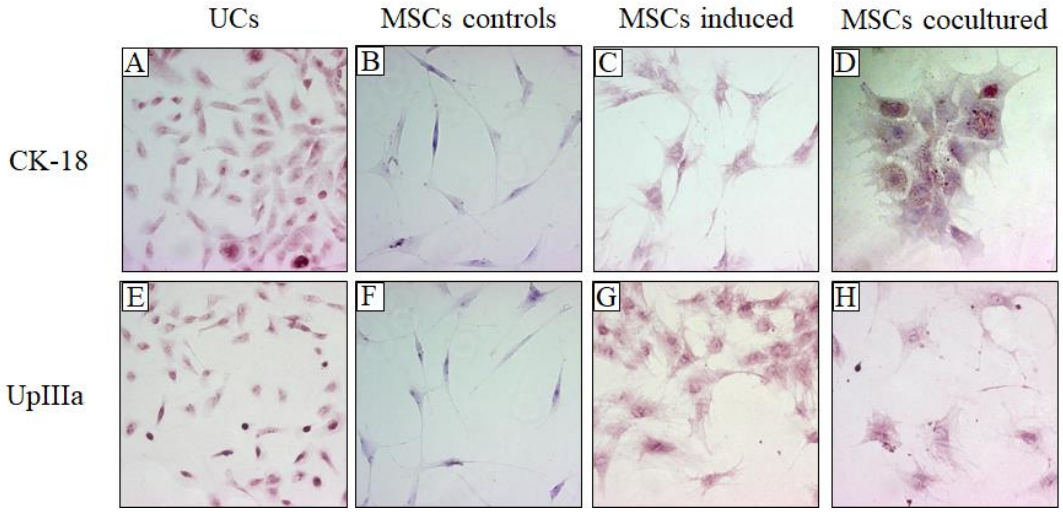

Figure 14: Characterization of induced MSCs with urothelial-specific markers after 14 days. The induced cells were positive after induction in CM (C,G) and after co-culture (D, H). UCs were used as positive controls (A,E) and non-induced MSCs were used as negative controls (B, F). Adapted from Scientific paper III, CC-BY 4.0.

RT-PCR confirmed the amplification of urothelial lineage gene transcripts of UpIIIa and CK-18, which were upregulated with both induction methods but not the controls. Western blot confirmed upregulated protein expression of UpIIIa and CK-18 was distinctly higher in the induced cells compared with the controls. CK-18 expression was higher in mesenchymal stem cells induced with conditioned medium, than after induction with co-culture.

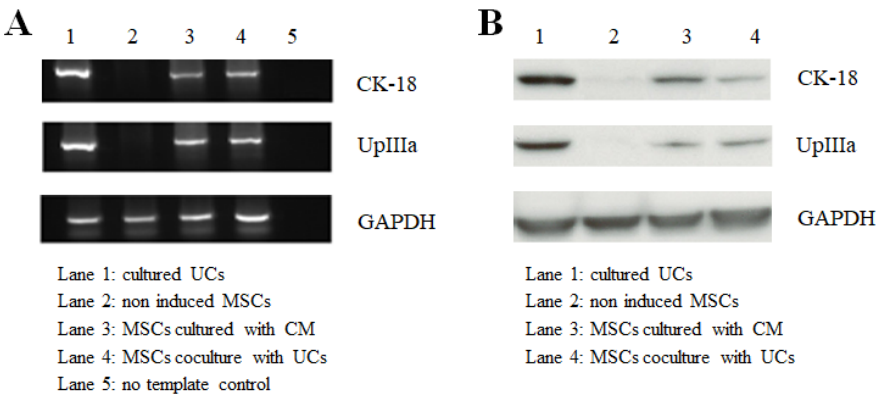

Figure 15: Investigation with RT-PCR (a) and western blot (b) confirmed differentiation to a urothelial lineage, by positive results of gene transcription and protein expression of CK-18 and UpIIIa. Adapted from Scientific paper III, CC-BY 4.0.

3.3.4 Induced mesenchymal stem cells on PCL-collagen scaffold

Routine histology verified an epithelial-like confluent cell layer on top of our scaffold in the induced MSC samples. Immunostaining was positive for MNF-116, CK-18 and UpIIIa in the induced samples and positive controls but not in our non-induced controls.

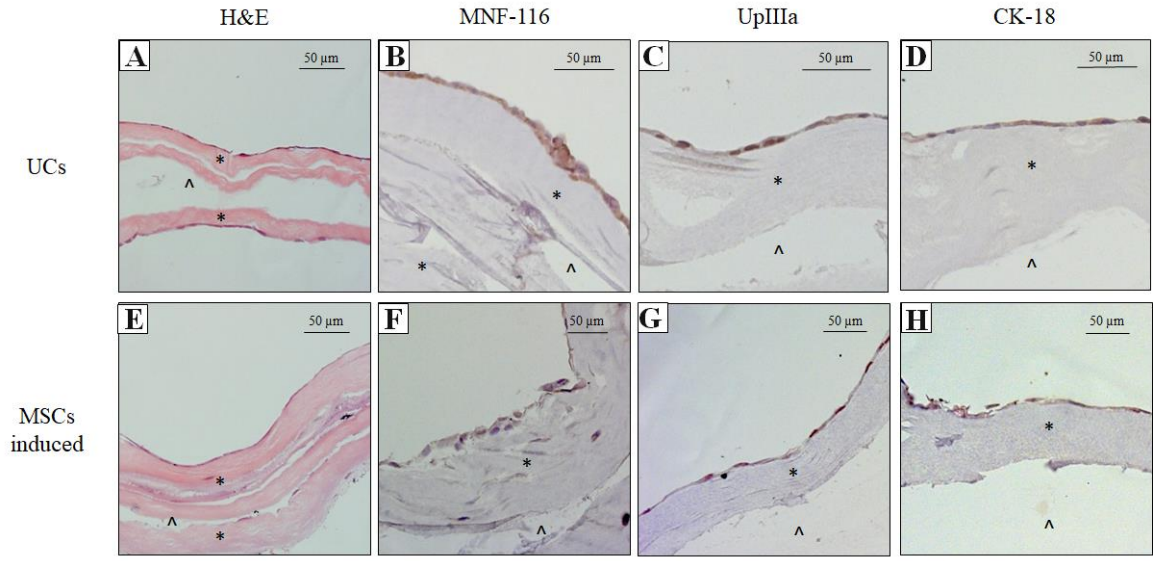

Figure 16: A comparison between urothelial cells and induced mesenchymal stem cells after 14 days. The arrowheads indicate the placement of the PCL and the asterisks indicate the collagen. UCs were used as positive controls (A-D) and induced MSCs were positive for epithelial marker MNF-116 (F) and urothelial specific markers UpIIIa (G) and CK-18 (H). Adapted from Scientific paper III, CC-BY 4.0.

3.4 STUDY IV

3.4.1 *In vitro* results

Macroscopically, tissue transplants showed no contraction and no delamination occurred between the PCL and collagen. Histological staining with H&E demonstrated that a single layer epithelium had been formed after two weeks, multilayered epithelium was formed after four weeks, and after six weeks a keratinized stratified squamous epithelium was visible *in vitro*.

3.4.2 Tensile strength

The transplants kept a high tensile strength throughout the experiment but lost some of their elasticity. The highest possible measured strength was 2.4 MPa and most transplants were intact at this point. The tensile strength was reduced from a median of 2.4 MPa at the start of the experiment to a median of 2.2 MPa after ten days and with higher variability, but after four weeks regained its former strength to 2.4 MPa. The elasticity of the construct decreased over time. The elongation or elongation at break was virtually unchanged from a median of 185% at the start of the experiment to 184% at ten days but with higher variability, and had been reduced to 123% after four weeks.

3.4.3 *In vivo* results

All rats survived the course of the study period, did not demonstrate any complications, and appeared unaffected by the implants. A total of 11 rats were operated with 24 transplants containing minced tissue and 39 transplants with no tissue. A total of 48 samples from the three time points (5, 10, 28 days) were collected for histology and 15 samples were collected for tensile strength measurements. Tissue growth could be observed already after five days and we could observe cells growing from borders of the minced tissue to cover the collagen (Figure 17). Clear multilayer epithelium resembling keratinized stratified squamous epithelium could be seen at ten days. None of the controls demonstrated epithelial growth. Immunohistochemistry confirmed a skin keratinocyte origin (Figure 18).

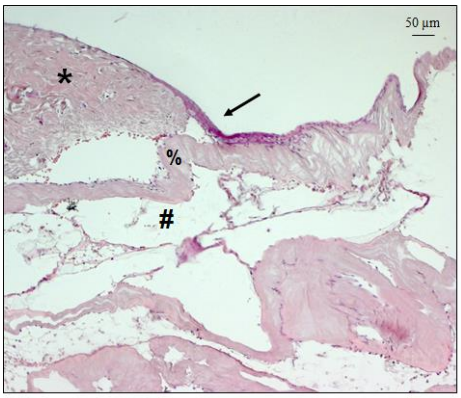

Figure 17: Newly regenerated tissue from the transplanted minced particles. Cells (arrow) have proliferated from the edge of the minced tissue (*) and covered parts of the collagen (%) and PCL (#) scaffold. Day five after transplantation. (H&E)

Integration was evaluated by cell ingrowth, visible capillaries, and the loosening and thinning of the collagen. The epithelium grew in local areas on top of the collagen and the transplants were better integrated when epithelium was present. The integration of the collagen had started at five days and was in most parts integrated after ten days and after four weeks the collagen was fully integrated in most areas. PCL fibers could be observed unchanged during the whole study period in all samples.

Inflammatory changes with redness were seen macroscopically in one of the samples (1/ 48) and confirmed microscopically with penetration of inflammatory cells within the transplant.

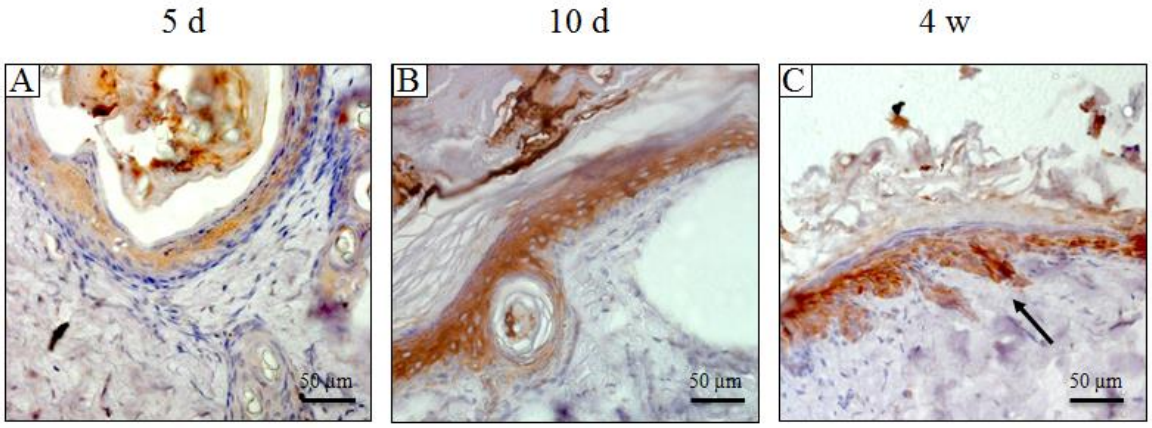

Figure 18: Figure demonstrating neo-epithelialization at different time points after transplantation. A: Single layer keratinocytes as seen after five days, B: Keratinized stratified squamous epithelium after ten days and, C: stratified squamous epithelium with increase of keratinization and development of mature interactions to the sub-epithelial tissue with epidermal ridges and dermal papilla (arrow) at four weeks. (C11 and HTX)

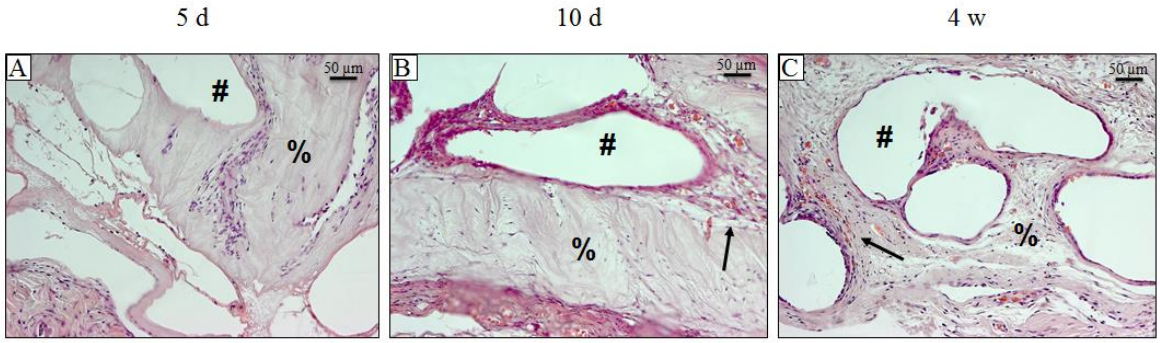

Figure 19: Figure demonstrating the interactions between the transplant and surrounding native tissue at different time points. A and B: Penetration of cells within the collagen (%) and reorganization of the collagen was seen already after five and ten days. Capillaries (arrow) within the scaffold were formed after 10 days. C: Collagen was fully integrated after 4 weeks. PCL (#) was unchanged after 4 weeks. (H&E)

4 DISCUSSION

In these studies, we have tried to go beyond the traditional tissue engineering where one expands tissue *in vitro* before transplantation and instead find a way to use our own bodies as incubators for the regeneration of tissue. Our objective was to develop an easily assembled off-the-shelf scaffold using U.S. Food and Drug Administration (FDA) approved materials that could be used in an ordinary surgical setting to expand tissue. We identified that such a scaffold needs to provide a beneficial environment for the cells and have the strength to sustain surgical handling and manipulation. However, it also needs to keep its strength over time to allow the take of the transplants, and tissue regeneration and maturation into functional tissue with characteristics that would fulfill the demands related to the intended application. We also have explored the possibility of inducing stem cells into urothelium on top of our scaffold to also provide a solution for patients with a total lack of tissue.

We used collagen type I, which is a part of, and resembles, the body's own extracellular matrix for the most substantial part of our scaffold. Collagen is known to enhance cell proliferation, activity and orientation, as well as exhibiting low inflammatory and antigenic responses. It also contributes to different cellular interactions by incorporating cell-adhesion peptide sequences, thus making it suitable for tissue engineering (Furthmayr and Timpl 1976, Silver and Pins 1992, Cen, Liu et al. 2008). The tensile strength became greater (0.6±0.12 MPa) by performing plastic compression of collagen. It would still not be enough for bladder augmentation purposes because of the bladder's high tensile strength (1.9 ±0.2 MPa) (Martins, Filho et al. 2011). Another disadvantage was the constriction which appeared when we cultured minced tissue on top of only collagen and made the plastic compressed collagen lose its form and dimensional shape. The next step was therefore to incorporate a polymer inside of our constructs for additional strength.

Two different polymers were used, PCL which yielded a tensile strength of 17.9 ±2.6 MPa and PLGA with a tensile strength of 3.6 ±1.1 MPa. Both polymers have the strength to withstand the tensions a bladder is subjected to until new tissue is formed and can withstand the regular forces of bladder filling and emptying. The measurements in our *in vivo* experiment with a PCL-collagen scaffold demonstrated a tensile strength up to 2.4 MPa, which is within the normal magnitude for healthy human bladders (Martins, Filho et al. 2011). The scaffold retained its high tensile strength over a long period and was still elastic over the observed four-week period. These qualities could make the scaffold suitable for tissue engineering of organs that need to be elastic, such as the urinary tract and tendons, for example the diaphragmatic muscle tendon (Schechtman and Bader 1997). In applications where higher elasticity is important, a dynamic or static extension of the scaffold may yield an even higher elasticity over time.

The polymers and the scaffold should preferably slowly degrade over time and be absorbed by the body as new cells, vessels and extracellular matrix are formed. The initial weakness of the transplant will come from its edges and we would most likely need to drain the bladder during the initial healing phase.

A common issue with our scaffolds was the attachment between the collagen and the different polymers. Our first attempts at combining the PCL and collagen resulted in unreliable constructs where the collagen would de-attach. We believed this was due to the hydrophobic nature of the PCL surface. After alkaline hydrolysis and treatment with PVA as previously described for similar cases (Pattison, Wurster et al. 2005), the PCL-mesh became more hydrophilic, which yielded an integrated construct. The same issues arose when trying to integrate collagen and PLGA. This time, we thought it depended on the pore size, and by increasing the pore size to around 3 times the collagen could integrate into the PLGA-mat. Part of the reason for this is the plastic compression which presses the collagen in between the PLGA fibers. Larger pore sizes may also lead to easier infiltration and migration of cells inside the transplant in an *in vivo* setting. When working with these materials we also found that the risk for collagen to polymer dehiscence decreased with larger sized transplants.

Our PCL- and PLGA-based scaffolds seeded with minced tissue either on top or inside the collagen showed a successful proliferation and migration of urothelial cells. We also observed a migration from cells both on top and inside the constructs. The cells reorganized on top of both types of scaffolds and after two weeks a proliferative monolayer had been formed. A multilayer epithelium was visible already after four weeks and was positive for both MNF-116 and Ki-67. This indicates the cells have an epithelial origin and are proliferative during the whole experiment. Cell division was mostly located in the lower layers of the multilayered epithelium, representating a basal membrane and resembling a transitional urothelium.

Even though the cell culturing properties of our scaffolds are similar, several differences are present between the PCL and PLGA scaffolds. The PCL has a higher tensile strength of 17.9 compared with 3.6 MPa for the PLGA and is easier to handle surgically. The PLGA on the other hand has a faster degradability than PCL; six months compared with two years (Gunatillake and Adhikari 2003). The PLGA was also possible to produce as a thinner mat, in our studies around 200 µm compared with 400 µm for the PCL-mesh, which would introduce less amount of synthetic material inside the body. Despite these differences, the results of both scaffolds are representative and it shows that neither the PCL nor the PLGA has a clear advantage. Even though *in vivo* studies may later prove a clinical advantage, the use of either polymer can be decided for other reasons if the previously stated differences are not highly important for the intended application. Other practical reasons for choosing either polymer may be the availability and the price. In Studies III and IV we opted for the PCL-collagen hybrid scaffold because of easier access to PCL.

With patients lacking autologous urothelium, such as after cancer treatment, severe trauma or mutilating surgery, minced mucosa may not be available for tissue engineering purposes as previously described. In these cases, multipotent stem cells may provide a solution. Mesenchymal stem cells are multipotent cells which reside in many places of the body. Mesenchymal stem cells are suitable for tissue engineering purposes because of their advantageous properties, such as high differentiation potential, anti-inflammatory, non-

immunogenic properties and low oncogenic risk (Pittenger, Mackay et al. 1999, Bartholomew, Sturgeon et al. 2002, Hare, Traverse et al. 2009, Prockop, Brenner et al. 2010). Other advantages are the ease of isolation of mesenchymal stem cells, which are already used in clinical trials, and the possibility to differentiate mesenchymal stem cells into ectodermal and endodermal lineages as well as a mesodermal lineage (Petersen, Bowen et al. 1999, Sanchez-Ramos, Song et al. 2000, Oswald, Boxberger et al. 2004, Le Blanc, Frassoni et al. 2008, Hare, Traverse et al. 2009, Connick, Kolappan et al. 2012).

Other groups have been able to use stem cells from adipose tissue and bone marrow and differentiate these into urothelial-like cells by exposure to a certain microenvironment (Tian, Bharadwaj et al. 2010, Ning, Li et al. 2011, Shi, Fu et al. 2012). We showed the possibility to differentiate adult mesenchymal stem cells into urothelial-like cells, using urothelium acquired with a minimally invasive method. Through bladder washings, urothelial cells were collected and the patient only had to undergo catheterization. This gives us a secure and easy way to collect urothelial cells for our differentiation purposes, and should therefore strongly facilitate the recruitment of donors.

The mesenchymal stem cells could be differentiated to urothelium through both conditioned medium and co-culturing. These results suggest that a substance is produced by the proliferating urothelial cells which leads the stem cells into a urothelial lineage. In theory, mesenchymal stem cells in the blood stream could therefore have a role in bladder wound healing. Here an autocrine and paracrine cell signaling could take place through the cell medium, but we did not investigate a juxtacrine model. Even though juxtacrine signaling may yield better results, but in our clinical scenario, autologous urothelial cells will not be available and we will not be able to use juxtacrine signaling for differentiation.

To evaluate our differentiation method for future clinical implementation we examined the differentiation of mesenchymal stem cells on top of a PCL-collagen based scaffold suitable for the urinary system. We could show a good attachment and proliferation of cells, as well as differentiation of mesenchymal stem cells on top of the hybrid scaffold after forming a confluent epithelial cell layer. In a clinical setting, urothelial cells from different donors would be frozen and stored to be used two weeks before the intended surgery with our transplant.

At this point we did not intend to incorporate the scaffold to the urinary bladder of a small animal model, in our case the rat, due to its small bladder size. To evaluate the integration and regeneration of a transplant it is crucial to evaluate a critical sized defect that cannot be healed in the body by contraction or ingrowth solely from the borders. A small-sized bladder defect would therefore not have given us results representative of human bladders. Therefore, we preferred to evaluate basic characteristics of the scaffolds such as: integration, tolerance and tensile strength in a small animal model as a first step. In addition, as we would only be able to harvest a small amount of bladder tissue for autologous transplantation to the same animal (only one tissue-loaded transplant per animal) that would also lead to a higher number of animals needed for the study.

The rational for using a small animal model was to evaluate the composite biomaterial including minced autologous epithelium after *in vivo* transplantation. During this step, where we only wanted to study tissue integration and epithelial tissue expansion, we could use a rodent model and decrease the number of large animals needed within the project.

Before our *in vivo* experiment (Study IV), we evaluated the scaffold with minced skin *in vitro* to make sure our scaffold was also feasible with skin. Analogous with bladder mucosa, we found that a single layer epithelium had formed after two weeks, a multilayer epithelium after four weeks and a keratinized stratified squamous epithelium was visible after six weeks *in vitro*.

The results of our *in vivo* studies showed that our scaffolds were totally integrated with surrounding tissue after four weeks, and were already integrated after ten days, as demonstrated by vascularization within the construct. We observed epithelial cells on top of the scaffold after five days, which demonstrates reorganization of minced tissue by migration and early proliferation of cells from minced tissues to the surface of the scaffold. A mature keratinized stratified squamous epithelium was present after four weeks. Our three-dimensional scaffold kept its tensile strength during the whole experiment and kept most of its elasticity despite the static conditions prevailing in the present experimental model.

We can now describe basic data that help us estimate changes in scaffold behavior *in vivo* over time. Integration of the scaffold takes approximately four weeks; this data is helpful for future *in vivo* studies where we aim at integrating the scaffold into the urinary system in a large animal model. We also confirmed that it would be possible to expand epithelial cells *in vivo* by placement on top of the scaffold prior to transplantation. We also observed a higher integration rate where the epithelial cells were located. These findings allow us to estimate how much time the scaffold needs to integrate inside the body, with or without minced tissue during the healing process.

In our pilot study, we used a rodent full-thickness skin wound model for the experiments, by these means the scaffolds were placed in the bottom of skin wounds that were held moist and covered with dressings as described in previous studies (Hackl, Bergmann et al. 2012). Unfortunately, the rats could escape their dressings and damage the transplants. We also noticed a pronounced contraction of the rat skin that would disturb further evaluation of the neo-regeneration around the transplant itself. Despite applying methods described in previous studies we could not prevent the constriction of the skin and the transplants were affected (Bae, Bae et al. 2012). We then decided to evaluate the transplant subcutaneously and not in a full skin wound model. In this model, the rats could not influence the transplant by scratching or biting and therefore the scaffold properties could be better evaluated. In addition, the subcutaneous implantation did not seem to disturb the rats as did the full-thickness wounds, which included a postoperative dressing. The downside of this modified model was that epithelial cells could not expand and cover all the scaffold surface, most likely due to the lack of discrepancy between air and tissue to guide proliferation. Therefore, as expected, the epithelial cells grew in clusters or in smaller areas on top of the collagen.

When deciding on our experimental setup we concluded that a skin wound model would be the most ethical way to proceed. Even though our main goal was to find a scaffold suitable for bladder augmentation a skin model required fewer rats and less morbidity for the animals. Previous studies have shown that a bladder rat model is not best suited for assessing how well a transplant will behave in large animal or human models (Sloff, Simaioforidis et al. 2014). This might be caused by the difference in bladder size and size of defect; a smaller bladder and wound may heal better because of easier cell migration and tissue contraction instead of relying on neo-regeneration. Therefore, in future studies we will allow for a critical size defect in our next experimental model that is comparable with human bladders.

5 CONCLUSIONS

5.1 STUDIES I AND II

We were able to construct a suitable scaffold for minced mucosa which can bypass the laborious, expensive and time consuming process of *in vitro* culture for tissue engineering, and instead have the possibility to expand tissue inside our own bodies. Our new hybrid scaffold consists of collagen as an outer part and is strengthened in the middle with a PCL mesh or a PLGA mat. The mechanical properties improved significantly when introducing PCL or PLGA into the scaffold, and the tensile strength was at the same magnitude or more than the bladder. Collagen and both polymers integrated well after specific optimization. In our *in vitro* studies, we saw the scaffold provide the minced tissue with an advantageous environment for cell proliferation and migration. With SEM and histology, we could observe a single layer epithelium covering the collagen after two weeks and a multilayered epithelium after four weeks. With these new scaffolds, we envision a clinical scenario where the surgeon can perform a bladder augmentation during one single surgical procedure with the patient's own mucosa, and the time between tissue collection and a finished transplant would be 30-40 min. Because the tissue is autologous there is no risk of rejection. This method could be performed in smaller hospitals lacking advanced laboratories and could make tissue engineering available for more patients.

5.2 STUDY III

For patients with a total lack of tissue, conventional tissue engineering or expanding tissue with minced pieces is not possible. In these cases, we can use mesenchymal stem cells collected from the bone marrow as a cell source for tissue engineering. We could differentiate mesenchymal stem cells into urothelial-like cells after two weeks in a microenvironment consisting of the growth medium of proliferating urothelial cells. Mesenchymal stem cells were also able to differentiate on top of a collagen-PCL based scaffold suitable for tissue engineering of the urogenital tract. Further studies are needed to explore the molecular mechanisms and evaluate the differentiated stem cells *in vivo* before a clinical trial could take place.

5.3 STUDY IV

By adding minced tissue to a collagen gel including a PCL-knitted fabric, cell expansion and re-organization of the epithelium could take place without the need for conventional *in vitro* cell culturing. Transplant properties seemed favorable over time *in vivo*. The method was simple and could be used as a one-stage procedure in an ordinary surgical unit for tissue expansion and could be implemented for bladder augmentation. The next step would be to evaluate the transplant with an autologous bladder epithelium in a large animal model.

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